Development of collagen-EDC scaffolds for skin tissue engineering: physicochemical and biological characterization

María Luisa Del Prado Audelo[1], Karla Karina Gómez Lizárraga[2], David Mauricio Giraldo Gómez[3], Héctor Martínez Hernández[4], Nayeli Rodríguez Fuentes[5], Andrés Eliú Castell Rodríguez[6], Edgar B. Montufar[7], María Cristina Piña Barba[8]

[1-5,8]Instituto de Investigaciones en Materiales, Universidad Nacional Autónoma de México, Ciudad Universitaria, Circuito Exterior S/N, Del. Coyoacán, C.P. 04510, México D.F., México.

[6]Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, Circuito Exterior S/N, Del. Coyoacán, C.P. 04510, México D.F., México.

[7]Central European Institute of Technology, Brno University of Technology (CEITEC-BUT), Brno, Czech Republic.

Abstract*—A leading consequence of burns is the loss of large extensions of skin. Thus, skin tissue engineering has been increased and promoted development of biomimetic skin scaffolds. Type I collagen is one of the most materials used in tissue engineering due to its biological characteristics. However, the applications of collagen as biomaterial are severely limited by its reduced physicochemical and mechanical properties, such as high susceptibility to enzymatic degradation in vivo and low thermo stability. To enhance collagen properties, crosslinked collagen scaffolds at different concentrations of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were prepared by freeze-drying technique. The effect of crosslinking and concentration on scaffolds physicochemical and biological behavior was evaluated. Scaffolds morphology was observed by Scanning Electron Microscopy, showing in all cases an appropriate microstructure for biological applications. Differential Scanning Calorimetric showed an increase in shrinkage temperature (TS) with increase in EDC concentration. Infrared Spectroscopy suggested that the secondary structure of collagen is not affected after the crosslinking. Enzymatic degradation test indicated that scaffolds treated with EDC dissolved slowly in enzymatic solution (just 12% of degradation after 96 h). Cell viability and attachment tests suggested that EDC treatment do not affect the excellent biological characteristics of collagen.*

***Keywords*— collagen, crosslinking, enzymatic degradation, freeze-drying, skin.**

I. INTRODUCTION

Skin is the largest organ of the body and it is composed of epidermis, dermis, and hypodermis layers, together with a complex nerve and blood supply systems [1], [2]. Loss of large part of skin, related to illness or injury, would not only affect the appearance of the patient, but also can lead to infection and even causing death. Thus, the necessity of skin substitutes for wound healing has increased skin tissue engineering research and promoted development of biomimetic skin scaffolds that help to regenerate large points of damaged skin [3], [4].

Collagen has been widely used to fabricate scaffolds due to its high biocompatibility, low antigenic response and because it naturally contains cell adhesion motives that improve cell-scaffold interactions [5]–[8]. Type I collagen is the most common type of collagen and it is the major protein of all connective tissue, such as bone, tendon, cartilage and skin [9]–[12]. Despite the huge efforts and developments, the uses of collagen for tissue engineering applications is currently limited by its high susceptibility to enzymatic degradation and low thermo stability *in vivo* [13]–[15]. For the development of new skin tissue engineering therapies, the enhancement of thermal, mechanical and enzymatic stability of collagen is needed. The crosslinking is the most used method collagen properties [8], [16], [17].

Glutaraldehyde has been extensively used as crosslinker, but it is associated with cytotoxicity effects, reduced cellular ingrowth *in vitro* and *in vivo* [13], [17], [18]. The search of "green" crosslinkers that avoid denaturation protein and can be easily removed after the crosslinking is the topic of current research in the field. Among potential alternatives, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) has demonstrated to be a mild[8], [19] and water-soluble crosslinker. EDC induce the formation of covalent amine bonds thought carboxyl and amino groups of collagen[8], [19], [20]. Usha *et.al.*[19] reported the physicochemical behavior of collagen crosslinked with EDC (10 mM). However, they do not report the biological performance of the scaffolds obtained. In contrast, Krishnamoorthy *et.al*. [21] report physicochemical and biological behavior of collagen crosslinked with EDC, but they use only one EDC concentration (40

mM). EDC is considered as a zero length crosslinker. Nevertheless, some authors have questioned about the cytotoxicity of EDC and the facility of the technique [22]. Thus, comprehensive study, about physicochemical and biological properties, of collagen crosslinked scaffolds with different concentrations of EDC is needed to assess the modification of collagen structure and its effects on cell viability.

The objective of this study was to access the effects of several EDC concentrations, if any, on the physicochemical and biological properties *in vitro* of collagen scaffolds for skin tissue engineering. The scaffolds were prepared by freeze-drying technique, crosslinked with different concentrations of EDC and characterized in terms of morphology, thermal stability, molecular structure, enzymatic degradation and cell attachment, viability and proliferation.

II. MATERIALS AND METHODS

2.1 Extraction of type I collagen

Type I collagen was isolated from fresh bovine Achilles tendon by acetic acid extraction and pepsin digestion method. Briefly, the minced tendons were suspended in 0.5 M acetic acid solution during 48 h, the viscous collagenous material was separated from the insoluble components by sieving and digested with pepsin (1g pepsin/ 100 g tendon) at 4°C during 12 h. Trypsin digestion was halted by 0.2 M sodium phosphate dialysis. The dialyzed collagen was centrifuged at 12000 g for 40 min to get rid of insoluble impurities. The supernatant was precipitated by NaCl (5 w/v %) and the precipitated was re-dissolved in 0.5 M acetic acid. Then, the solution was dialyzed with 4 mM potassium phosphate buffer to repeat the same process to achieve the protein purification. Finally, collagen was re-dissolved in 0.5 M acetic acid and the collagen solution was stored at 4°C. The purity of the collagen extracted was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed following the method of Laemmli [23] using discontinuous Tris/HCl/Glycine buffer system.

2.2 Fabrication and crosslinking of collagen scaffolds

Freeze-drying technique was used for fabrication of the collagen scaffolds. Briefly, the collagen solution (5 mg/ml) was dropped onto silicon molds (diameter: 30 mm, depth: 10 mm) and frozen at -20 °C for 24 h. After that, the frozen collagen was lyophilized at -40 °C and 0.05 mBar for 18 h. For crosslinking the lyophilized scaffolds were immersed in 5 ml of EDC-ethanol solutions with different concentrations of EDC (0, 35mM, 45mM and 55mM) during 24 h at 4 °C. The crosslinked scaffolds were washed three times with distilled water during 10 minutes using a sonicator (Branson) at room temperature. The scaffolds were freeze-dried again applying the same conditions derived above. Non-crosslinked scaffold (0 mM EDC) was named COL and was prepared as a control to could compare the changes in the protein after the crosslinking. Changes in the protein structure after EDC crosslinking were analyzed by Fourier transform infrared spectroscopy (FTIR Nicolet 6700) from 4000 cm^{-1} to 400 cm^{-1}.

The ultrastructure of the scaffolds was observed by field emission scanning electron microscope (JEOL JSM-7600F). The crosslinked collagen scaffolds were cut along its diameter and the cross-section was coated with a thin layer of gold (Fine Coat Ion Sputter JFC-1100) to prevent charging during observation. Pore size was determinate image analysis (AxioVision 4.0). The percentage of porosity was obtained applying equation 1.

$$Porosity\ (\%) = (1 - \rho^*/\rho) \times 100 \quad (1)$$

Where ρ^* is the apparent density of the scaffold determined by the ratio between the dry mass of the scaffold and its external volume, and ρ is the theorical density of collagen (1.3 g/ml [24]).

2.3 Swelling test

To determinate the absorption of water, the scaffolds were immersed in sealed tubes containing PBS and incubated at 37°C during 60 min. Afterwards, the scaffolds were removed, gently dry with filter paper to remove the excess of PBS, and the percentage of water uptake was calculated using equation 2:

$$Water\ uptake\ (\%) = \frac{W_{wet} - W_{dry}}{W_{dry}} \times 100 \quad (2)$$

Where W_{dry} is the weight of the sample and W_{wet} is the weight of the sample after immersion in PBS. The test was performed in triplicate.

2.4 Thermal stability of collagen-EDC scaffolds

The thermal stability of the EDC crosslinked collagen scaffolds was measured through differential scanning calorimetry (DSC 2910 Modulated TA Instruments).The thermograms were obtained by heating the samples from 0 to 120 °C with a heating rate of 5 °C/min.

2.5 Enzymatic Degradation

Enzymatic degradation of collagen-EDC scaffolds was performed using collagenase digestion. Scaffolds were cut into disks (superficial area), and were immersed in 2 ml of Tris-HCl buffer solution (pH 7.4) containing 5 U/ml of collagenase type I (*clostridium hystoliticum*, Sigma-Aldrich) at 37 °C. The solution was renewed every 8 h. Scaffold samples were removed after 12, 24, 48, 72 and 96 h of immersion, rinsed the with distilled water and lyophilized. The percentage of enzymatic degradation was calculated according to equation 3. All samples were tested in triplicate.

$$Mass\ loss\ (\%) = \frac{W_i - W_f}{W_i} \times 100 \quad (3)$$

Where, W_i is the weight of the scaffold before collagenase digestion and W_f is the weight of the scaffold after degradation.

2.6 Cell culture of human dermal fibroblasts

Primary human dermal fibroblasts were obtained from voluntary donors of the Faculty of Medicine, UNAM, following protocols reviewed and approved by Ethics committee of Faculty. The cells were cultured in Minimum Essential Medium (MEM; GibcoBRL), supplemented with 2 mM glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) maintaining the culture environment at 37°C and 5% CO_2. Prior cell seeding, scaffold disks (diameter: 6 mm, depth: 2 mm) were placed in tubes with PBS 1% penicillin/streptomycin overnight, sterilized by UV treatment for 1 hour (30 minutes each side) and pre-moistened in MEM during 24 h. Fibroblasts were seeded in the scaffolds using 96 well cell culture plates at a density of 1500 cells/well. After 24 h cell viability was evaluated using AlamarBlue reagent (Invitrogen) following the instructions of the producer [25]. Briefly, the culture medium was discarded and fresh medium with 10% of AlamarBlue was added. The color turnover was quantified by measuring the optic absorbance of 100µl of supernatant in a BioTek Epoch Microplate Spectrophotometer at 570 nm and 600 nm. The first measure was obtained immediately after the AB was added (time 0), the next measurements were performed at different times (6 h, 12 h, 1, 2, 4, 5, 6 and 8 days). The morphology of the fibroblasts attached to the scaffolds was evaluated after 3 h, 24 h and 7 days of proliferation. Scaffolds were rinsed three times with Na-cacodylate buffer, fixed in 4% glutaraldehyde solution, dehydrated in ethanol series and prepared for SEM observation applying a thin layer of gold to prevent charging.

2.7 Statistical analysis

Data were analyzed by one-way ANOVA to determinate statistical significance between groups, *p*-values <0.05 were considered as significant.

III. RESULTS AND DISCUSSION

3.1 Purity of type I collagen extracted from Achilles bovine tendon

Figure 1 shows a representative electrophoresis pattern of extracted collagen. The pattern of collagen shows two well differentiated bands at 130 and 140 kDa, correspondig to α1 and α2 [26] chains, respectively. Besides, in agreement with Phanat Kittiphattanabawon *et. al.* [27], the pattern shown a high molecular weight band (250 kDa) related with β components, which is considered a dimer of the α chains. The structure of type I collagen is formed by two α1 chains and one α2 chain ([α1(I)]2 α2(I)) [28]. These results suggested that the collagen isolated had the typical type I collagen structure and do not show any detectable degradation products.

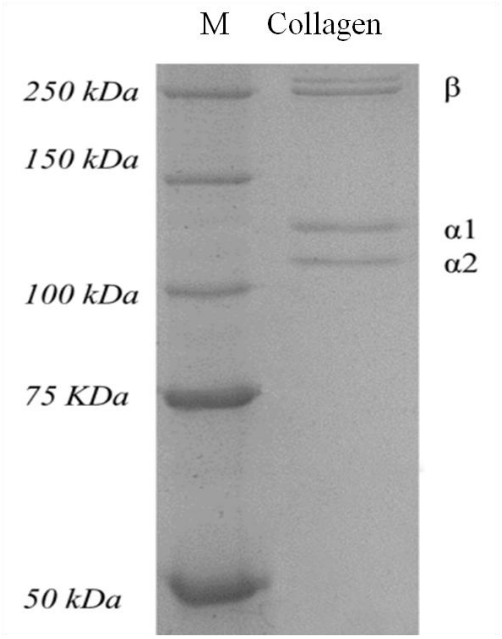

FIGURE 1. SDS-PAGE PATTERN OF M: PRECISION PLUS PROTEIN UNSTAINED STANDARDS BIO-RAD AND TYPE I COLLAGEN FROM *BOVINE ACHILLES TENDON*.

3.2 Collagen-EDC scaffolds characterization

SEM images of longitudinal cross sections of collagen-EDC scaffolds show highly porous structure in all samples (Fig.2). The scaffolds have a continuous network of open porous. Porosity was estimated around 99% for all scaffolds without significant differences between them (table 1). Furthermore, the pore size estimated from SEM images was similar for all scaffolds (table 1). Therefore, the structure of the scaffolds was not significantly affected by EDC crosslinking. Similar results were obtained by Davidenko *et.al.* [29]. The minimum pore size found was about 21 µm while the biggest one was 566 µm. Therefore, the pore size was within a range suitable for growth of cells, such as, fibroblasts, myocytes and endothelial cells [24], [29]. Accordingly, the morphology of all scaffolds can be considered as appropriate for their use in skin tissue engineering applications [30].

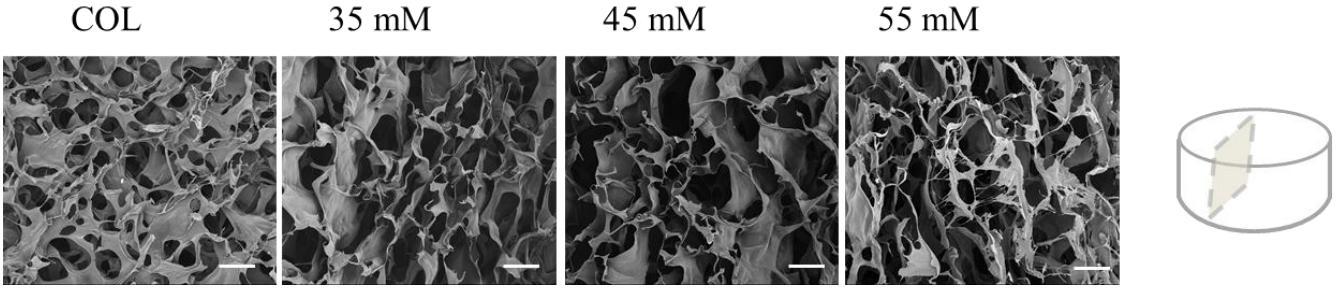

FIGURE 2. REPRESENTATIVE SEM IMAGES OF LONGITUDINAL CROSS SECTIONS OF COLLAGEN-EDC SCAFFOLDS (SCALE BAR OF 100 µM).

The results of PBS absorption are shown in Table 1. In general, crosslinked scaffolds show higher swelling ability than uncrosslinked scaffold (COL) which shows a PBS uptake about 2000%. This result is in accordance with the work of Martinez *et.al.*[24] and Skopinska-Winsniewska et.al. [22] who found, for native collagen, uptakes around 2000% and 2200%, respectively. The maximum PBS absorption was observed on 55 mM collagen-EDC scaffold. These properties of absorption are favorable for wound healing application.

TABLE 1
POROSITY AND PORE SIZE OF COLLAGEN-EDC SCAFFOLDS

Sample	Density (g/ml)	Relative density	Porosity (%)	Pore size (µm)	PBS uptake (%)
COL	9.5 E-6 ± 0.12 E-6	7.31 E-6 ± 0.09 E-6	99 ± 9.81E-6	115 ± 54	2050 ± 13.6
35mM	9.94 E-6 ± 0.26E-6	7.65 E-6 ± 0.2 E-6	99 ± 2.01E-5	110 ± 50	2764 ± 5.8
45mM	1.40 E-5 ± 0.02 E-5	1.08 E-5 ± 0.02 E-5	99 ± 2.121E-5	121 ± 72	8620 ± 3.7
55mM	1.28 E-5 ± 0.01E-5	9.89 E-6 ± 0.11 E-6	99 ± 1.106-5	108 ± 51	12656 ± 1.6

Mean ± SD

The influence of the EDC on swelling properties of scaffolds could be due to variances in hydrophilicity caused by reduction in lateral groups and the presence of new chemical bonds [31].

The FT-IR spectra for collagen-EDC scaffolds are shown in Fig. 3. In the uncrosslinked collagen spectrum (COL), the four typical collagen bands can be observed. Amide A band at 3300 cm^{-1} it is commonly associated with the N–H and O-H stretching vibrations (ν_{NH} and ν_{OH}) [32], [33]; Amide B was found at 3077 cm^{-1}. The Amide I band represents 80% of the stretching vibrations of the peptide carbonyl group ($\nu_{C=O}$) with some C-N stretching (10%) and N-H bending (10%), it is found at 1633 cm^{-1} [34], [35]. Amide II band at 1538 cm^{-1} and corresponds to N-H deformation vibrations (δ_{NH}) and C-N stretching (ν_{CN}) [33]. After EDC crosslinking, the wavenumber of collagen bands are almost the same as non-crosslinked samples (table 2). This may suggest that the secondary structure of collagen was not affected [36]. However, the intensity of bands varies with the EDC concentration.

TABLE 2
BAND POSITION (cm^{-1}) IN THE FT-IR SPECTRA OF COLLAGEN-EDC SCAFFOLDS

Sample	Amide A	Amide B	Amide I	Amide II	Amide III
COL	3300	3077	1633	1538	1237
35	3204	3078	1631	1538	1234
45	3300	3078	1633	1538	1235
55	3292	3077	1633	1538	1235
Band assignment	ν_{NH}, ν_{OH}	ν_{NH}	$\nu_{C=O}$, ν_{NH}	ν_{CN}, δ_{NH}	ν_{CN}, δ_{NH}

The Amide I band, which is the most useful infrared band for the analysis of the secondary structure of proteins, presented decrease on its intensity (Fig. 3B). This due to the formation of new covalent bonds because crosslinking. In addition, the characteristic carboxyl stretching, observed between 1440 and 1390 cm^{-1} [35] appear in COL spectrum (Fig. 3C), but decrease (almost disappear) in crosslinked-EDC scaffolds. This indicates that several carboxyl groups take part in crosslinking reaction. Although that no significant increment in the N-H bound was observed (Amide A band), the decrement of Amide I intensities suggest a more covalent crosslinked structure and weakened carboxyl group related to formation of new bonds. Moreover, this result is in agreement with the higher thermal stability of the crosslinked samples. Therefore, the stabilization effect of EDC on collagen could spread its applications in tissue engineering.

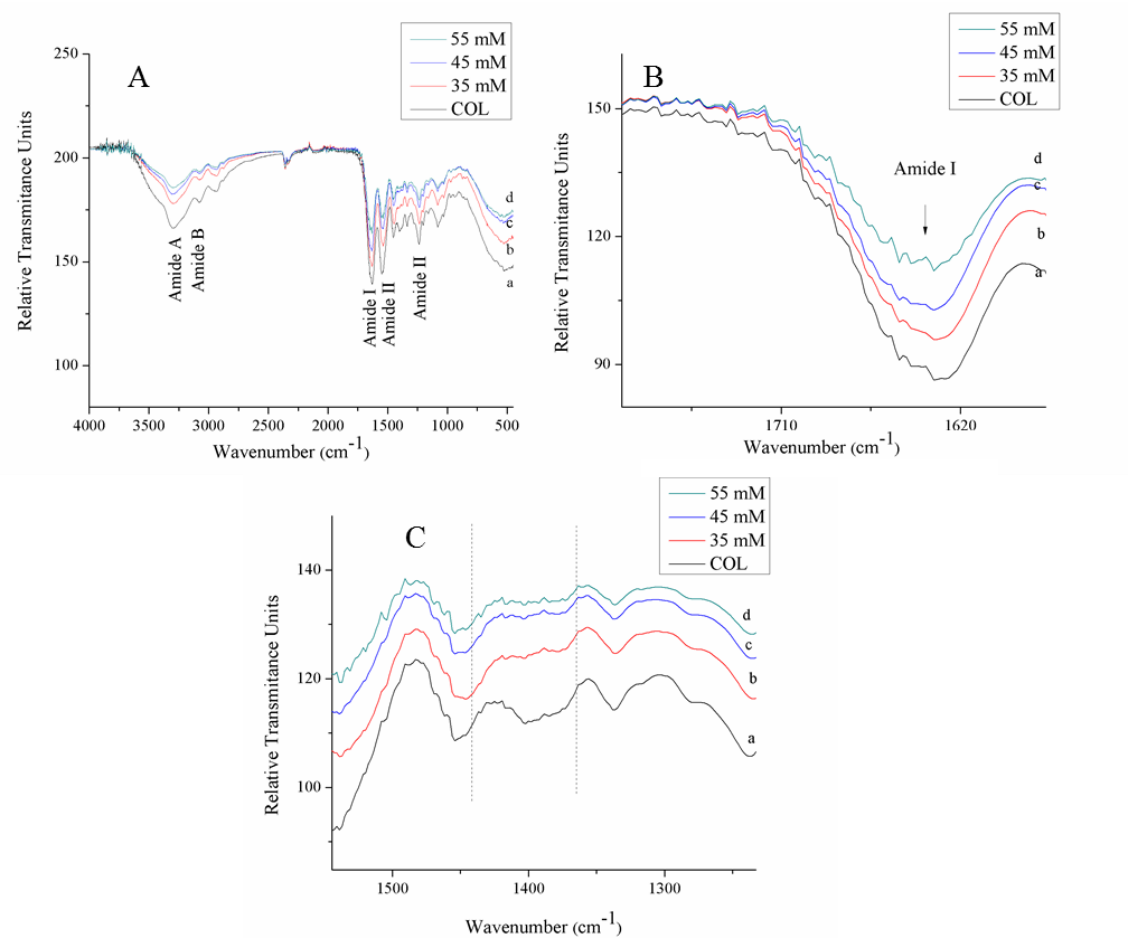

FIGURE 3. FT-IR SPECTRA OF COLLAGEN-EDC SCAFFOLDS. ALL SPECTRA SHOW TYPICAL COLLAGEN BANDS (A). THE INTENSITY OF AMIDE I BAND DECREASED AS CONCENTRATION OF EDC INCREASED (B). THE CARBONYL BAND DECREASED AS CROSSLINKING TAKE PLACE (C). COL LINE (a), 35 mM line (b), 45 mM line (c), 55 mM line (d).

DSC thermogram of collagen-EDC scaffolds crosslinked showed slight differences in shrinkage temperature (T_S) with increase in EDC concentration (Fig. 4). T_S was the highest for 55 mM (95°C), followed by 45 mM and 35 mM (both with 90°C), and finally by COL with 80 °C. T_S is considered as the temperature at which collagen denaturation begins, due to the rupture of interchain bonds causing the collagen shrinks [37], [38]. Consequently, this temperature has been defined as a measure of the structural stability of collagen [39].

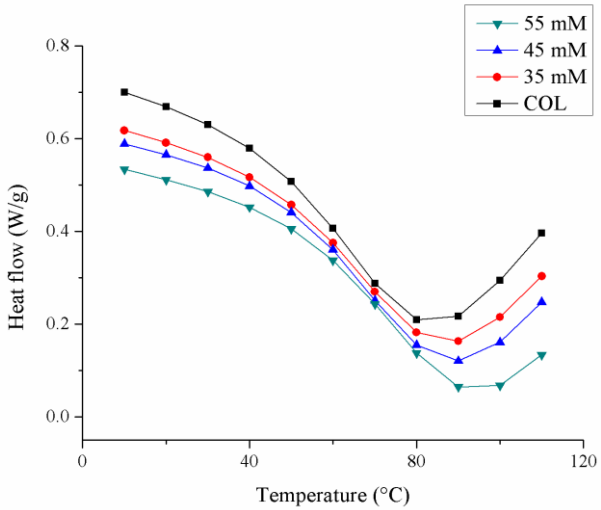

FIGURE 4. DSC THERMOGRAM OF COLLAGEN-EDC SCAFFOLDS.

For comparison, Krishnamoorthy et. al. [40] reported that collagen crosslinked with gallic acid obtained a value of T_S of 80 °C, this means that EDC gives a better thermal stability.

Due to the stabilization of triple helix in collagen structure by crosslinking, break these additional crosslinks requires more energy during heating. Then, it could be expected that collagen treated with EDC provides scaffolds with increased stability preventing the denaturation at low temperatures.

3.3 Enzymatic Degradation

Collagen is one of the most used materials in tissue engineering due to its excellent biocompatibility and weak antigenicity. However, native collagen presents a high enzymatic degradation rate, reducing its possible clinical applications [40], [41]. Enzymatic degradation of crosslinked collagen scaffolds was studied by monitoring the residual weight percent after different times of incubation with type I collagenase Tris-HCl buffer. Fig. 5 shows the weight loss (%) as a function of time. COL presented the lowest resistance to enzymatic degradation, dissolving it in just two hours after test started (result not shown). In contrast, scaffolds treated with EDC dissolved slowly in collagenase solution, presenting mainly 12, 17 and 46% of degradation after 96 hours respectively for 55, 45 and 35 mM collagen-EDC scaffolds.

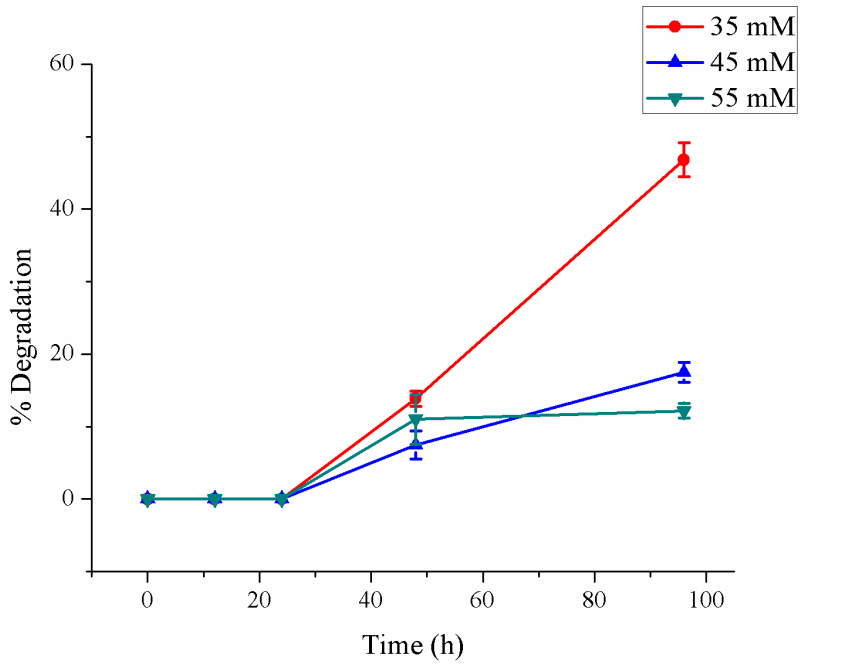

FIGURE 5. MASS LOSS (%) IN PRESENCE OF COLLAGENASE IN TRIS-HCL AT 37°C FOR COLLAGEN-EDC SCAFFOLDS.

Recently, different kind of crosslinkers has been used to decrease degradation rate of collagen. Skopinska-Wisniewska [42], used squaric acid as crosslinker. About 17% of the highest crosslinked scaffold was degraded by collagenase within 1 h. Other authors [40], used galllic acid as crosslinker on collagen scaffolds, and it was observed that, at 96 h on contact with collagenase solution, the scaffold with highest content of crosslinker presented 16% of degradation. Therefore, enhance the structural stability of collagen scaffolds with additional crosslinks by EDC crosslinking is a better and effective method to prevent the cleavage by collagenase and improve the characteristics of collagen as biomaterial.

3.4 Cell culture test

It is well accepted that cells be seeded and proliferated successfully in collagen scaffolds [43], [44]. Formaldehyde and Glutaraldehyde has been commonly used as crosslinkers. However, this kind of agents has been associated with cytotoxicity effect. Meyer et.al. [45] report the cytotoxicity effects in scaffolds crosslinked with Formaldehyde and 0.1% Glutaraldehyde. In both, it presented a highly cytotoxicity. As mentioned above, EDC is a zero-length crossliker, which means that the crosslinker will not part of the final product. Therefore, the use of EDC should present not cytotoxic results. However, it is

important to evaluate if EDC crosslinking method affects the citocompatibility of the scaffolds, this is usually done by viability and proliferation assays. The viability and proliferation of human dermal fibroblasts in presence of crosslinked collagen scaffolds were evaluated for 8 days of incubation using AB reagent. Results shown in Fig. 6 demonstrated that EDC treatment is not cytotoxic, showing equivalent cell viability than the non-crosslinked scaffold and no significant difference between the samples and the negative control. The negative control (cells with AlamarBlue medium, but without the presence of the scaffolds), show the lower cell population. These results suggest that the collagen of the scaffolds keep its excellent biological characteristics after the crosslinking method.

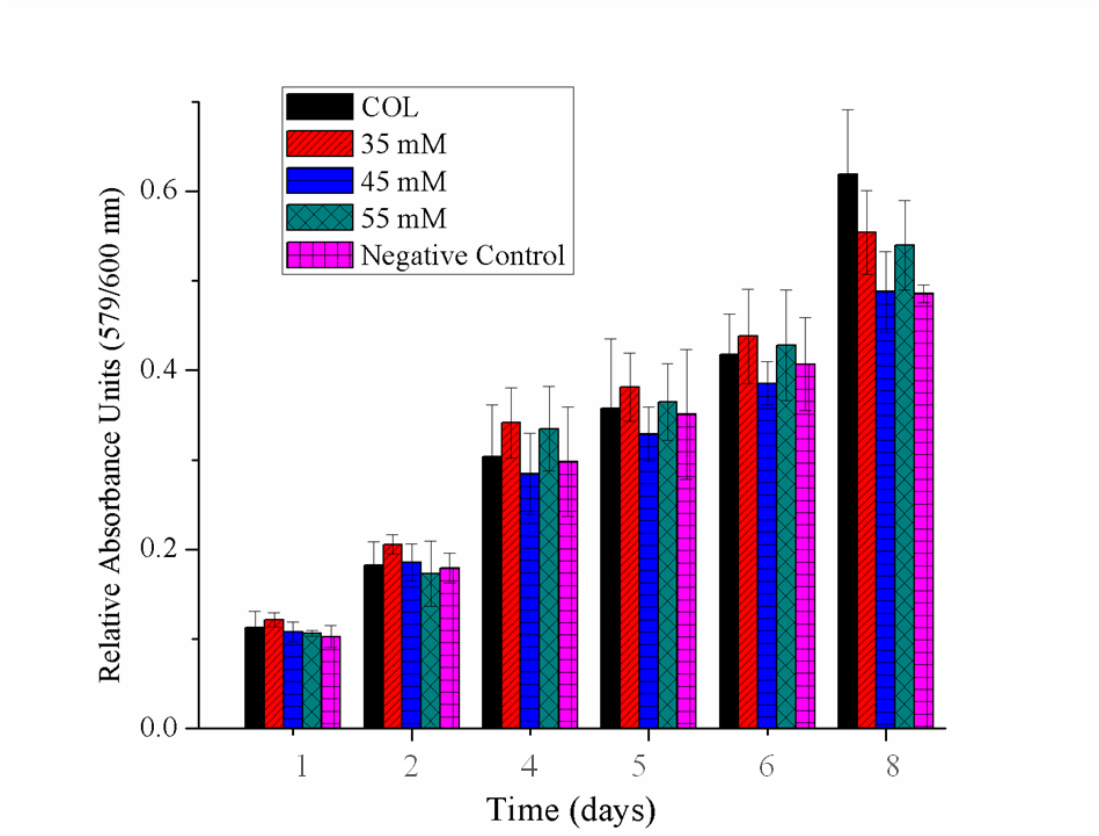

FIGURE 6. EFFECT OF COLLAGEN-EDC SCAFFOLDS ON HUMAN DERMAL FIBROBLASTS. CELL VIABILITY AND PROLIFERATION WAS EVALUATED BY ALAMARBLUE REDUCTION ALONG DURING 8 DAYS OF INCUBATION. THERE IS NO SIGNIFICANCE DIFFERENCE BETWEEN GROUPS (N=5)

The morphology of fibroblasts cultured on collagen-EDC a scaffold was examined by SEM. Images were collected at different times of culture (3h, 24h and 7 days). However, fibroblasts morphology on COL scaffolds was not observed due to low stability of the sample, which generates scaffold degradation and collapse. It is worth to mention that collagen presents good biological properties but, as commented above, has very low stability under biological conditions, being remarkable important to find methods that improve mechanical performance, such as EDC crosslinking. In fact, EDC crosslinked scaffolds, presented structural stability along the cell culture test, allowing imaging of the cells.

The cells within the collagen-EDC scaffolds showed spread morphology and cells colonize the three-dimensional structure of the scaffolds (Fig. 7). After 3 h of seeding, the cells showed the typical elongated morphology of fibroblasts in contact with the crosslinked scaffolds. Furthermore, cells presented cytoplasmatic extensions, through filipodia (FL), to link with the matrix; this result suggests that fibroblasts present a high affinity with crosslinked scaffolds.

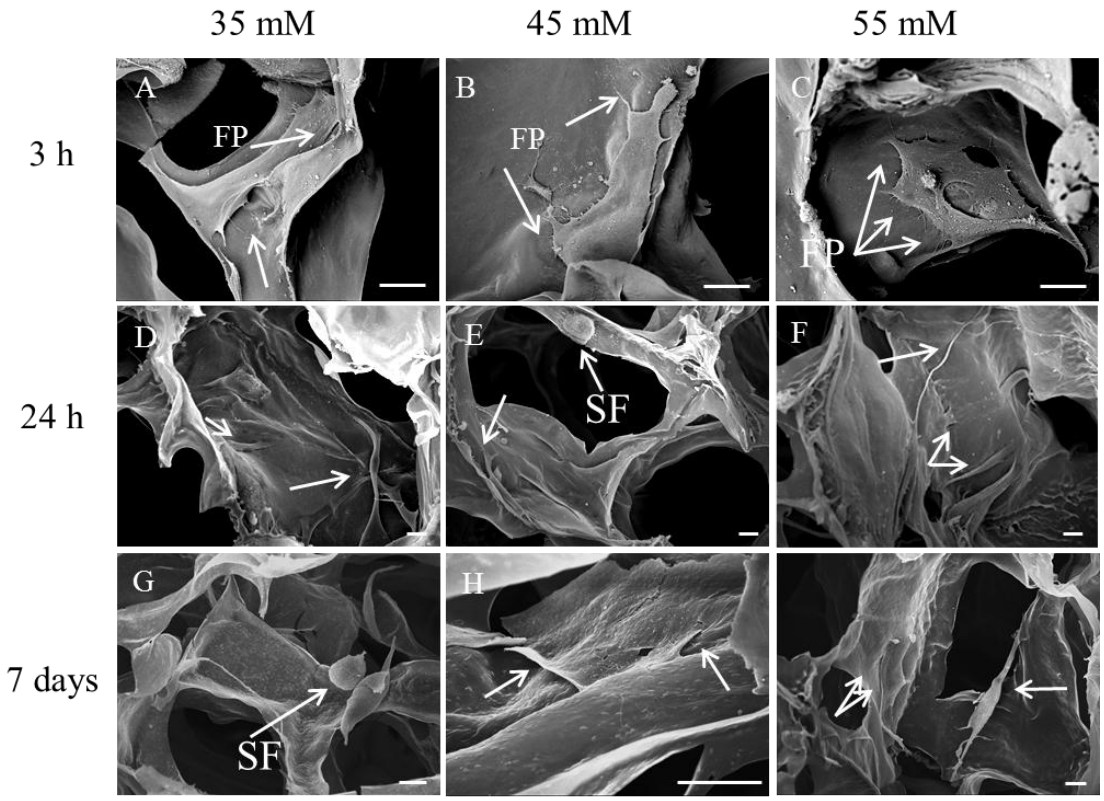

FIGURE 7. REPRESENTATIVE SEM IMAGES SHOWING THE MORPHOLOGY OF THE FIBROBLASTS CULTURED ON COLLAGEN-EDC SCAFFOLDS. AT 3 H OF SEEDING (A-C). AT 24 H OF SEEDING (D-F). AT 7 DAYS OF SEEDING (G-I). SCALE BAR 10 μM.

After 24 h of culture, cell density was increased. Cells cover almost all the sample surfaces presenting several attachment points with the scaffold. Furthermore, at 24 h, in some regions of scaffolds 35 and 55 mM it was possible to observe the synthesis of fibrous matrix (Fig. 8). These results corroborate that after crosslinking EDC the scaffolds are still biocompatible. Nonetheless, no significant effect on EDC concentration on cell attachment was found under the described experimental conditions. Fibroblast growing behavior and proliferation were analyzing after 7 days of culture. The formation of a thin cell layer was observed; these results demonstrated a good cell interaction with the surface of the collagen-EDC scaffolds. Cells in spheroidal form (SF) were also identified, this means that fibroblasts continued to proliferate after 7 days. Due to fibroblasts, the main cell type in dermis, showed high affinity to the collagen-EDC scaffolds, these are potential biomaterials for skin tissue engineering, specially the scaffold crosslinked at 55 mM of EDC, because it presented the higher thermal and enzymatic stability.

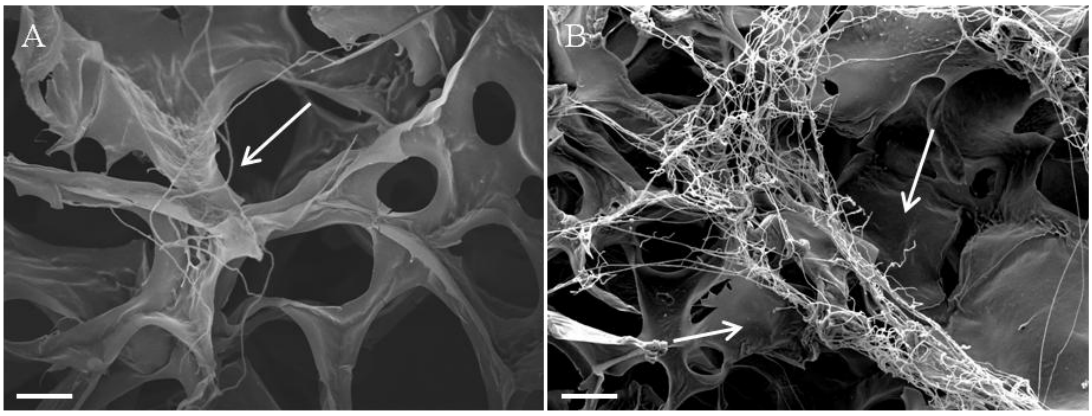

FIGURE 8. SEM MICROGRAPHS OF SYNTHESIS OF FIBROUS MATRIX IN A) 35 MM SCAFFOLD (SCALE BAR 20 μM) AND B) 55 MM SCAFFOLD (SCALE BAR 40 μM). ARROWS INDICATE THE PRESENCE OF MATRIX.

IV. CONCLUSION

Type I collagen scaffolds were successfully stabilized by crosslinking treatment with EDC. The results of the physicochemical characterization of the crosslinked scaffolds indicate that EDC treatment has not significantly altered the porous morphology of scaffolds, but improve the structural stability and thermal properties, necessary behavior in materials for tissue engineering applications. Scaffolds showed the same proliferation profile and before and after crosslinking treatment, besides the cells showed affinity for the crosslinked scaffolds. All these results suggest that this study could be useful for development a collagen-based biomaterial for tissue engineering applications having suitable physicochemical properties and reduced antigenicity, even with high EDC concentrations. The findings of this study are limited to the *in vitro* behavior. Furthermore, they are a starting point and the *in vivo* performance should be evaluated.

ACKNOWLEDGEMENT

The authors thank to Miguel Ángel Canseco, Armando Zepeda Rodríguez, Francisco Pasos Nájera and Josué Esaú Romero Ibarra for their technical assistance. This paper constitutes a partial fulfillment of the Graduate Program in Materials Science and Engineering at Universidad Nacional Autónoma de México (UNAM), Del Prado-Audelo M.L. acknowledges the scholarship provided by the Consejo Nacional de Ciencia y Tecnología (CONACyT No. 346323). This study was supported by DGAPA-UNAM trough the project: PAPIIT- IG100114 and CONACyT trough the project: 214128. EBM acknowledged the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II.

REFERENCES

[1] F. Groeber, M. Holeiter, M. Hampel, S. Hinderer, and K. Schenke-Layland, "Skin tissue engineering - In vivo and in vitro applications," Adv. Drug Deliv. Rev., vol. 63, no. 4, pp. 352–366, 2011.

[2] L. Cen, W. E. I. Liu, L. E. I. Cui, W. Zhang, Y. Cao, R. S. W. L, S. People, and S. J. Tong, "Collagen Tissue Engineering : Development of Novel Biomaterials," vol. 63, no. 5, pp. 492–496, 2008.

[3] L. Yildirimer, N. T. K. Thanh, and A. M. Seifalian, "Skin regeneration scaffolds: A multimodal bottom-up approach," Trends Biotechnol., vol. 30, no. 12, pp. 638–648, 2012.

[4] F. Wang, M. Wang, Z. She, K. Fan, C. Xu, B. Chu, C. Chen, S. Shi, and R. Tan, "Collagen/chitosan based two-compartment and bi-functional dermal scaffolds for skin regeneration," Mater. Sci. Eng. C, vol. 52, pp. 155–162, 2015.

[5] H. Tan, B. Wu, C. Li, C. Mu, H. Li, and W. Lin, "Collagen cryogel cross-linked by naturally derived dialdehyde carboxymethyl cellulose," Carbohydr. Polym., vol. 129, pp. 17–24, 2015.

[6] A. L. S. Padmanabhan, L. Salvatore, F. Gervaso, M. Catalano, A. Taurino, A. Sannino, "Synthesis and Characterization of Collagen Scaffolds Reinforced by Eggshell Derived Hydroxyapatite for Tissue Engineering - ResearchGate.pdf." Journal of Nanoscience and Nanotechnology, United States of America, pp. 1–6, 2014.

[7] M. H. Santos, R. M. Silva, V. C. Dumont, J. S. Neves, H. S. Mansur, and L. G. D. Heneine, "Extraction and characterization of highly purified collagen from bovine pericardium for potential bioengineering applications," Mater. Sci. Eng. C, vol. 33, no. 2, pp. 790–800, 2013.

[8] G. Krishnamoorthy, P. K. Sehgal, A. B. Mandal, and S. Sadulla, "Novel collagen scaffolds prepared by using unnatural D-amino acids assisted EDC/NHS crosslinking," J. Biomater. Sci. Polym. Ed., no. June 2014, pp. 1–21, 2012.

[9] J. A. M. Ramshaw, Y. Y. Peng, V. Glattauer, and J. a. Werkmeister, "Collagens as biomaterials," J. Mater. Sci. Mater. Med., vol. 20, no. SUPPL. 1, 2009.

[10] K. Matmaroh, S. Benjakul, T. Prodpran, A. B. Encarnacion, and H. Kishimura, "Characteristics of acid soluble collagen and pepsin soluble collagen from scale of spotted golden goatfish (Parupeneus heptacanthus)," Food Chem., vol. 129, no. 3, pp. 1179–1186, 2011.

[11] D. Liu, X. Zhang, T. Li, H. Yang, H. Zhang, J. M. Regenstein, and P. Zhou, "Extraction and characterization of acid- and pepsin-soluble collagens from the scales, skins and swim-bladders of grass carp (Ctenopharyngodon idella)," Food Biosci., vol. 9, pp. 68–74, 2015.

[12] M. Ogawa, R. J. Portier, M. W. Moody, J. Bell, M. A. Schexnayder, and J. N. Losso, "Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (Pogonia cromis) and sheepshead seabream (Archosargus probatocephalus)," Food Chem., vol. 88, no. 4, pp. 495–501, 2004.

[13] J. E. Gough, C. A. Scotchford, and S. Downes, "Cytotoxicity of glutaraldehyde crosslinked collagen / poly (vinyl alcohol) films is by the mechanism of apoptosis," 2001.

[14] C. Ding, M. Zhang, and G. Li, "Preparation and characterization of collagen / hydroxypropyl methylcellulose (HPMC) blend film," Carbohydr. Polym., vol. 119, pp. 194–201, 2015.

[15] J. Kozłowska and a. Sionkowska, "Effects of different crosslinking methods on the properties of collagen–calcium phosphate composite materials," Int. J. Biol. Macromol., vol. 74, pp. 397–403, 2015.

[16] K. Madhavan, D. Belchenko, A. Motta, and W. Tan, "Evaluation of composition and crosslinking effects on collagen-based composite constructs," Acta Biomater., vol. 6, no. 4, pp. 1413–1422, 2010.

[17] H. M. Powell and S. T. Boyce, "EDC cross-linking improves skin substitute strength and stability," Biomaterials, vol. 27, no. 34, pp. 5821–5827, 2006.

[18] A. Cooke, R. F. Oliver, and M. Edward, "An in vitro cytotoxicity study of aldehyde-treated pig dermal collagen.," Br. J. Exp. Pathol., vol. 64, no. 2, pp. 172–176, 1983.

[19] R. Usha, K. J. Sreeram, and a. Rajaram, "Stabilization of collagen with EDC/NHS in the presence of l-lysine: A comprehensive study," Colloids Surfaces B Biointerfaces, vol. 90, no. 1, pp. 83–90, 2012.

[20] S. A. Madison and J. O. Carnali, "PH optimization of amidation via carbodiimides," Ind. Eng. Chem. Res., vol. 52, no. 38, pp. 13547–13555, 2013.

[21] G. Krishnamoorthy, R. Selvakumar, T. P. Sastry, A. B. Mandal, and M. Doble, "Effect of d-amino acids on collagen fibrillar assembly and stability: Experimental and modelling studies," Biochem. Eng. J., vol. 75, pp. 92–100, 2013.

[22] J. Skopinska-wisniewska, K. Olszewski, A. Bajek, A. Rynkiewicz, and A. Sionkowska, "Dialysis as a method of obtaining neutral collagen gels," Mater. Sci. Eng. C, vol. 40, pp. 65–70, 2014.

[23] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4.," Nature, vol. 227, no. 5259, pp. 680–685, 1970.

[24] A. Martínez, M. D. Blanco, N. Davidenko, and R. E. Cameron, "Tailoring chitosan/collagen scaffolds for tissue engineering: Effect of composition and different crosslinking agents on scaffold properties," Carbohydr. Polym., vol. 132, pp. 606–619, 2015.

[25] B. Pierce, "AlamarBlue Cell Viability Assay Reagent Thermo Scientific," 2012.

[26] S. Benjakul, Y. Thiansilakul, W. Visessanguan, S. Roytrakul, H. Kishimura, T. Prodpran, and J. Meesane, "Extraction and characterisation of pepsin-solubilised collagens from the skin of bigeye snapper (Priacanthus tayenus and Priacanthus macracanthus)," J. Sci. Food Agric., vol. 90, no. 1, pp. 132–138, 2010.

[27] P. Kittiphattanabawon, S. Benjakul, W. Visessanguan, T. Nagai, and M. Tanaka, "Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (Priacanthus tayenus)," Food Chem., vol. 89, no. 3, pp. 363–372, 2005.

[28] M. H. Uriarte-Montoya, J. L. Arias-Moscoso, M. Plascencia-Jatomea, H. Santacruz-Ortega, O. Rouzaud-Sández, J. L. Cardenas-Lopez, E. Marquez-Rios, and J. M. Ezquerra-Brauer, "Jumbo squid (Dosidicus gigas) mantle collagen: Extraction, characterization, and potential application in the preparation of chitosan-collagen biofilms," Bioresour. Technol., vol. 101, no. 11, pp. 4212–4219, 2010.

[29] N. Davidenko, C. F. Schuster, D. V. Bax, N. Raynal, R. W. Farndale, S. M. Best, and R. E. Cameron, "Control of crosslinking for tailoring collagen-based scaffolds stability and mechanics," Acta Biomater., vol. 25, pp. 131–142, 2015.

[30] L. Ma, C. Gao, Z. Mao, J. Zhou, J. Shen, X. Hu, and C. Han, "Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering," Biomaterials, vol. 24, no. 26, pp. 4833–4841, 2003.

[31] P. Velmurugan, R. R. Jonnalagadda, and B. U. Nair, "Biochemical and biophysical characterization of EDC treated rattus type i collagen," Process Biochem., vol. 48, no. 7, pp. 1059–1064, 2013.

[32] A. Barth, "Infrared spectroscopy of proteins," Biochim. Biophys. Acta - Bioenerg., vol. 1767, no. 9, pp. 1073–1101, 2007.

[33] H. Staroszczyk, K. Sztuka, J. Wolska, A. Wojtasz-Pająk, and I. Kołodziejska, "Interactions of fish gelatin and chitosan in uncrosslinked and crosslinked with EDC films: FT-IR study," Spectrochim. Acta - Part A Mol. Biomol. Spectrosc., vol. 117, pp. 707–712, 2014.

[34] B. D. C. Vidal and M. L. S. Mello, "Collagen type I amide I band infrared spectroscopy.," Micron, vol. 42, no. 3, pp. 283–9, 2011.

[35] B. H. Stuart, Infrared Spectroscopy: Fundamentals and Applications, vol. 8. 2004.

[36] A. Sionkowska, J. Skopinska-Wisniewska, M. Gawron, J. Kozlowska, and A. Planecka, "Chemical and thermal cross-linking of collagen and elastin hydrolysates," Int. J. Biol. Macromol., vol. 47, no. 4, pp. 570–577, 2010.

[37] A. J. Kuijpers, G. H. M. Engbers, J. Krijgsveld, S. a. J. Zaat, J. Dankert, and J. Feijen, "Cross-linking and characterisation of gelatin matrices for biomedical applications," J. Biomater. Sci. Polym. Ed., vol. 11, no. 3, pp. 225–243, 2000.

[38] K. Nam, T. Kimura, and A. Kishida, "Physical and biological properties of collagen-phospholipid polymer hybrid gels.," Biomaterials, vol. 28, no. 20, pp. 3153–62, 2007.

[39] T. C. H. H. C. W. Stringer, "The shrinkage temperature of skin collagen," Aust. J. Dermatol., vol. 5, no. 4, pp. 230–234, 1960.

[40] G. Krishnamoorthy, R. Selvakumar, T. Parvathaleswara, S. Sadulla, A. Baran, and M. Doble, "Experimental and theoretical studies on Gallic acid assisted EDC / NHS initiated crosslinked collagen scaffolds," Mater. Sci. Eng. C, vol. 43, pp. 164–171, 2014.

[41] L. Sang, D. Luo, S. Xu, X. Wang, and X. Li, "Fabrication and evaluation of biomimetic scaffolds by using collagen – alginate fi brillar gels for potential tissue engineering applications," Mater. Sci. Eng. C, vol. 31, no. 2, pp. 262–271, 2011.

[42] J. Skopinska-wisniewska, J. Kuderko, A. Bajek, M. Maj, A. Sionkowska, and M. Ziegler-borowska, "Collagen / elastin hydrogels cross-linked by squaric acid," Mater. Sci. Eng. C, vol. 60, no. 32, pp. 100–108, 2016.

[43] L. Chen, Z. Xiao, Y. Meng, Y. Zhao, J. Han, G. Su, B. Chen, and J. Dai, "The enhancement of cancer stem cell properties of MCF-7 cells in 3D collagen scaffolds for modeling of cancer and anti-cancer drugs," Biomaterials, vol. 33, no. 5, pp. 1437–1444, 2012.

[44] T. Zhou, N. Wang, Y. Xue, T. Ding, X. Liu, X. Mo, and J. Sun, "Development of Biomimetic Tilapia Collagen Nanofibers for Skin Regeneration through Inducing Keratinocytes Differentiation and Collagen Synthesis of Dermal Fibroblasts," ACS Appl. Mater. Interfaces, vol. 7, no. 5, pp. 3253–3262, 2015.

[45] M. Meyer, H. Baltzer, and K. Schwikal, "Collagen fi bres by thermoplastic and wet spinning," Mater. Sci. Eng. C, vol. 30, no. 8, pp. 1266–1271, 2010.